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Mechanisms of aluminum-induced microcytosis: Lessons from accidental aluminum intoxication

CARLOS A. CARAMELO, JORGE B. CANNATA, MIGUEL R. RODELES, JOSÉ LUIS FERNÁNDEZ MARTÍN, JUAN R. MOSQUERA, BEATRIZ MONZÚ, JULIO OUTEIRIÑO, GUILLERMINA BLUM, CONCEPCIÓN ANDREA, ANTONIO J. LOPEZ FARRÉ, GONZALO ACUÑA, SANTOS CASADO, and LUIS HERNANDO

Fundación Jiménez-Díaz and Unidad de Investigación, Universidad Autónoma and Universidad de Oviedo, Instituto Reina Sofía de Investigación Nefrologíca, Madrid-Oviedo, Spain

Mechanisms of aluminum-induced microcytosis: Lessons from accidental aluminum intoxication. Twenty-three hemodialysis patients exposed to an accidental aluminum overload, showed increased erythropoietin requirements and decreased erythrocyte mean corpuscular volume (MCV). At the peak of the intoxication, MCV and plasma aluminum levels changed from unrelated ($r = 0.02$) to strongly related ($r = 0.425$) variables. The molar proportion of plasma aluminum to plasma iron increased dramatically (from 1:13.8 to 1:2.4). This significant increment in the aluminum/iron ratio made higher the relative offer of aluminum with respect to iron to the erythroid precursor cells. Accordingly, in a subset of 13 randomly selected aluminum-intoxicated patients we found increased intraerythrocytic aluminum, which paralleled the increase in plasma aluminum. Furthermore, in the aluminum-intoxicated group, intraerythrocytic ferritin, a marker of iron content, and the ratio between erythrocyte and plasma ferritin were lower ($P < 0.01$ and <0.001, respectively), than in the control group. These findings support the hypothesis that in some cases of aluminum-related microcytosis, a ferropenic mycrocitosis, as expression of erythroid ferropenia, may exist in spite of the presence of normal body iron stores.

Aluminum-related microcytosis was first recognized in groups of patients undergoing dialysis therapy with improperly prepared water [1–4]. Since then, as a consequence of the generalization of adequate water treatment this complication decreased rapidly; however, aluminum intoxication still needs to be ruled out as a cause of anemia or resistance to erythropoietin in dialysis patients [5, 6].

The current concept about the pathogenesis of aluminum-related microcytosis is that aluminum might interfere with iron kinetics at different levels, including alterations in delta amino levulinic dehydrase activity and intermediate porphyrin products [7–11], and impaired intestinal iron absorption and cellular uptake [12].

In the summer of 1993 we had a unique opportunity to examine a group of patients treated in an independent, extra-Hospitalary dialysis facility, who presented an important, but not massive, increase in plasma aluminum levels. During a seven-month observation period, data were obtained which illustrate, in an unique situational model, several issues of human aluminum intoxication. These data may contribute to understanding the pathogenetic mechanism of aluminum-related microcytic anemia.

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Methods

Patients

In July 1993, a failure in the water treatment system of an independent dialysis facility, which consisted of an inadequate connection of the reverse osmosis system, was identified. The failure allowed some amount of non-treated water to reach the patient's dialysis bath. Until this exposure, the current policy was to check the patient's and dialysis fluid's aluminum concentrations every six months. The last aluminum determination of dialysis fluid was <10 μg/liter, and the aluminum concentration in the dialysis fluid by the time of detection of the failure was 44.7 ± 3.4 μg/liter. Therefore, even though the exact length of the exposure is difficult to determine, it could have lasted for a maximum of six months.

In phase 1 (see below), we studied an initial group of 23 patients (15 males and 8 females), who were exposed to the high aluminum concentration. During the time of the study, no significant changes in the treatment were introduced, except for increments in the dose of erythropoietin needed to achieve an hematocrit of around 30%. No patient had received aluminum-containing phosphate binders at any time for the previous two years. The 23 aluminum-exposed patients did not show classical symptoms of aluminum intoxication, with the exception of a few observations of lesions of porphyria cutanea; these are the subject of a separate study (Mosquera et al, unpublished data). In view of the results obtained by comparing the data at time I with those at time II, further studies were done in a randomly assigned subgroup of 13 patients (Phase 2), as detailed below, comparing them with an age- and sex-matched control group formed by 10 patients of the University Hospital who were dialyzed during the treatment time with a dialysis fluid containing less than 10 μg/liter of aluminum.

The biochemical and hematological data were obtained by routine methods (SMAC-20 and Coulter S plus VI). Transferrin was measured by an immunonephelometric method (Behringwerke A.G., Marburg, Germany) and total iron-binding capacity was calculated from this measurement. Iron was determined by the Ferrozine/Guanidine method (Hoffman La Roche, Basel,

Switzerland). Aluminum was measured as described in previous reports [12–14] by atomic absorption spectrometry in a specially prepared clean room Class 100. The detection limit of the method was 1 μg/liter, and the coefficient of variation was lower than 3% [12–14].

Intraerythrocytic aluminum was measured in red cells obtained from heparinized blood using aluminum-free tubes and pipettes washed and centrifuged twice with saline buffer, to eliminate blood cells other than erythrocytes. Erythrocytes were lysed using an aqueous solution containing Tryton X-100 (1%) and EDTA (1 mM), and aluminum was measured in the lysate. No significant amounts of aluminum were detected in the vehicles; heparin was used for blood sampling.

Ferritin determinations were done using a double antibody radioimmunoassay (Nichols Institute, San Juan Capistrano, California, USA). In phase 2, in the subgroup of 13 patients, we simultaneously measured ferritin in plasma and erythrocytes by a modification of previously described methods [15, 16]. Blood was treated similarly as described in the paragraph above, except that routine plastic tubing was used.

Design of the study

Phase 1. Three sets of laboratory data were compared, corresponding to: (I) three months previous to the aluminum exposure, in which the aluminum content of the dialysis water was <10 μg/liter; (II) at the time of the detection and repair of the failure in the water treatment system (maximum aluminum exposure); and (III) four months after the repair of the water treatment system. During this latter period, the patients were dialysed against an aluminum concentration bath of less than 10 μg/liter.

Phase 2. Additional determinations were performed in a smaller, randomly selected group of 13 patients. These determinations of intracellular ferritin and intracellular aluminum were, however, done after one month of treatment against a low aluminum bath. Since the present study was organized in the emergency circumstances of the sudden discovery of an aluminum intoxication, no preparatory arrangements could be done to perform a systematic study of all the possibly relevant variables in all the patients. Furthermore, it was ethically mandatory to repair the technical failure in the shortest time possible. These circumstances may introduce some limitations in interpreting the results of erythrocyte aluminum and ferritin.

Data analysis

Results are presented as mean ± SEM. Changes in variables were analyzed by one-way and two-way analysis of variance for repeated measures and subsequent Scheffé test. Comparisons between two groups of data were done by Student's *t*-test for paired and unpaired observations (two tails). Comparisons between two numerical variables were done by regression analysis and slopes were compared by *t*-test when appropriate. A *P* value of <0.05 was considered significant.

Results

Phase 1

From period I to period II there was a significant increase in serum aluminum levels from 31.9 ± 4.6 to 147.3 ± 11.7, $P < 0.0001$, and a decrease in MCV from 88.3 ± 1.5 to 81.5 ± 1.7, both $P < 0.0001$. In the same periods no significant change was found in plasma iron (82.7 ± 11.8 to 72.3 ± 6.5), plasma transferrin (283.3 ± 17 to 251.6 ± 11.2), iron transferrin saturation (31.7 ± 4.04 to 30.8 ± 3.0), or serum ferritin (494 ± 160 to 404.3 ± 129.8). In addition, to maintain a constant hematocrit (Period I: 30.6 ± 0.5, Period II: 30.4 ± 0.6), we also observed a significant ($P = 0.0047$) increment in the requirements of human erythropoietin (from 5542 ± 722 units in Period I to 7344 ± 910 units to period II). In period I there was no relationship between MCV and serum aluminum ($r = 0.02$); by contrast, in period II, there was a significant inverse relationship between both parameters, ($P < 0.001$, Fig. 1). Four months after the correction of the water treatment failure (Period III), an additional comparison between serum aluminum and MCV was performed. As it can be also observed in Figure 1, the group had partially corrected their values, even though they still had significant differences ($P < 0.001$) with respect to the baseline MCV/aluminum relationship. When we analyzed on a molar basis, the proportion of both metals in serum, we found a significant increase in the Al/Fe ratio in period II with respect to period I (Fig. 2).

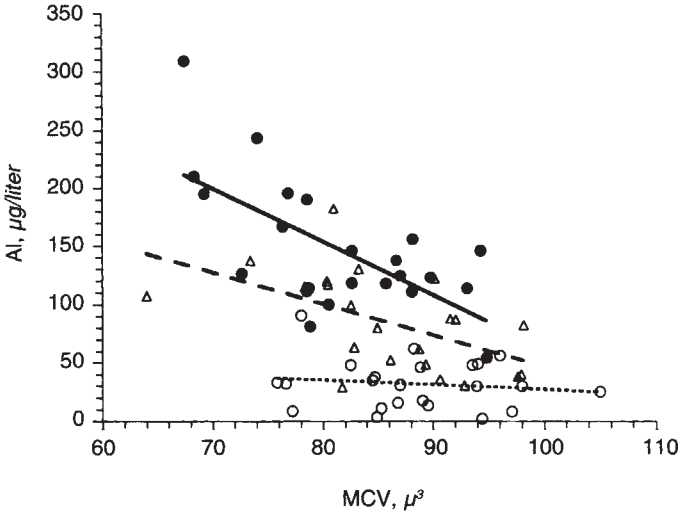

Fig. 1. *Phase 1.* Relationship by regression analysis between plasma aluminum levels and MCV at three different times: before aluminum intoxication (⊖, period I); at the time of detection of aluminum intoxication (●, maximum Al levels, period II); and four months after the returning to normal aluminum concentrations (△, <10 μg/L, period III) in the dialysis fluid. The regression lines corresponding to times II and III were significantly different with respect to period I ($P = 0.008$).

Phase 2

The comparative data of intraerythrocytic and plasma aluminum and ferritin in the subset of 13 patients are shown in Table 1. The intraerythrocytic and serum aluminum levels were significantly higher in aluminum-intoxicated patients in whom a significant negative relationship persisted between these two parameters and MCV (intraerythrocytic vs. MCV $r = 0.371$, $P = 0.027$; serum Al vs. MCV $r = 0.393$, $P = 0.021$). No statistical relationship was found between these parameters in the control group. In addition, the levels of intraerythrocytic ferritin were significantly lower ($P < 0.05$) in the aluminum-intoxicated patients (Table 1). When expressed in attog/cell, those values were 2.2 ± 0.5 and 5.3 ± 0.7 for aluminum-intoxicated and control patients, respectively ($P = 0.023$). The ratio between intraerythrocytic and

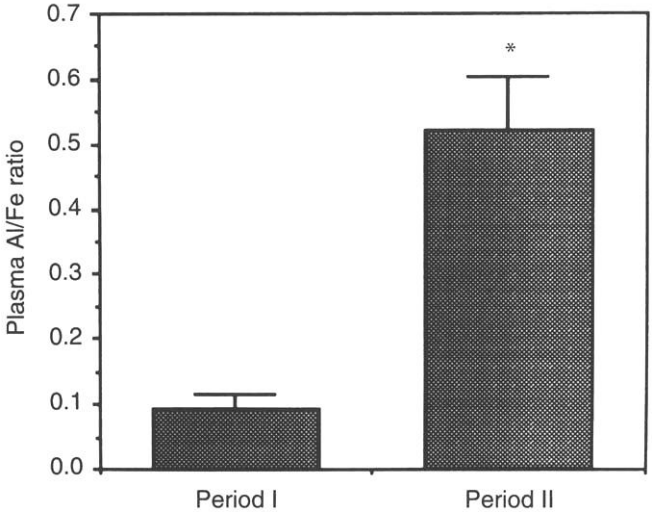

Fig. 2. *Phase 1.* Plasma Al/Fe ratio before and after the detection of aluminum load (period I 0.093 ± 0.021 vs. period II 0.521 ± 0.083). * $P <$ 0.0001 between periods I and II.

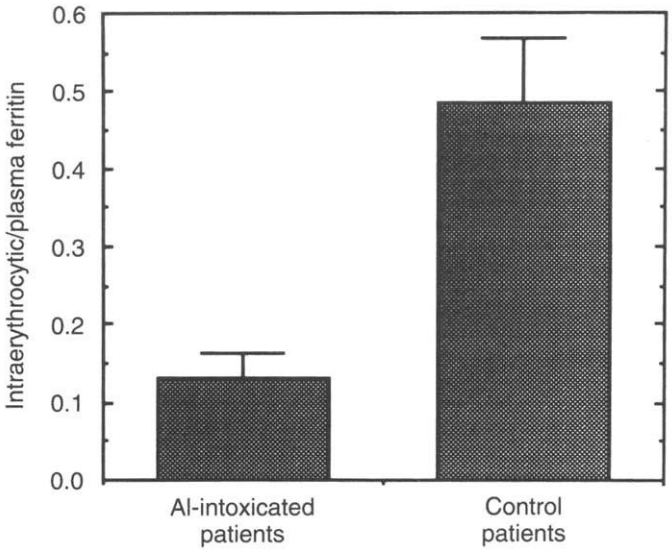

Fig. 3. *Phase 2.* Intraerythrocytic and plasma ferritin ratio in aluminum-intoxicated patients (N = 12) and controls (N = 10). P = 0.03 between groups. For this comparison, ferritin was expressed as ng/ml of plasma and ng/ml packed red blood cells (Al-intoxicated patients 0.131 ± 0.032 vs. control patients 0.486 ± 0.081).

Table 1. Phase 2

	Al intoxicated patients	Control patients	P
Erythrocyte Al *μg/kg*	133.1 ± 14.4	31.08 ± 3.2	<0.0001
Plasma Al *μg/liter*	110.2 ± 11.6	26.2 ± 4.26	<0.0001
Erythrocyte ferritin *ng/ml*	33.4 ± 4.01	93.3 ± 24.7	0.012
Plasma ferritin *ng/ml*	338.6 ± 149	167.1 ± 52.7	NS

Comparative data of intraerythrocytic and plasma aluminum and ferritin in 13 aluminum-intoxicated patients and 10 controls. All P values correspond to differences between control and aluminum-intoxicated patients.

plasma ferritin showed a marked decrease in aluminum-intoxicated patients compared with controls (Fig. 3).

Discussion

The occurrence of an accidental aluminum intoxication in a Dialysis Unit offered a unique opportunity for studying the interactions between aluminum and iron kinetics in a evolving situation in human subjects. The reports published to date on the role of aluminum as a cause of microcytic anemia correspond to series of patients treated chronically with high aluminum-containing dialysate and, therefore, little comparison could be done with previous baseline controls in the same patients [2–4]. This fact made difficult to analyze the pathogenetic conditions involved in the appearance of microcytosis. In addition, most of the previous studies corresponded to the pre-rHuEPO era.

Short, Winney and Robson have proposed that anemia may be the first manifestation of aluminum overload [2]. More recently, aluminum overload was related by other authors with the appearance of augmented needs of rHuEPO [5, 6]. Our findings are in agreement with these previous reports, showing the appearance of microcytosis and a significant increase in the rHuEPO requirements after a period of aluminum overload.

The main observations in the patients analyzed herein include the hematological differences found after the aluminum exposure. The highly correlated relationship between MCV and plasma aluminum, the higher intraerythrocytic aluminum with respect to plasma aluminum, and the lower intraerythrocytic ferritin compared with plasma ferritin, are the three main findings which allow us to interpret the pathogenetic sequence of the microcytic anemia of these patients.

Plasma ferritin level is a well-known marker of the total magnitude of iron stores, but it is not as good marker of the amount of iron offered to the erythroid precursors [14–16]. The measurement of the intraerythrocytic ferritin in the present study has permitted an additional insight in the process of iron acquisition by the erythroid precursors in conditions of aluminum intoxication.

The hypothesis that aluminum might cause anemia by preventing iron from binding or unloading from transferrin was originally raised in 1983 by Trapp [17]. The data obtained here show that the aluminum load did not have a specific effect in reducing the capacity of transferrin to transport iron, but it probably altered the offer of iron to its target cells, namely, the erythrocyte precursors.

High transferrin saturation by iron has been viewed as a limitating factor for increasing aluminum transport by the same transferrin molecules. In previous studies, Cannata et al have found a significant inverse relationship between serum iron and transferrin saturation versus serum aluminum in 127 dialysis patients. However, it is important to note that sharing sites for plasma iron and aluminum transport without one metal displacing the other should be possible every time that an incomplete iron transferrin saturation exists [10].

Cellular transferrin uptake occurs mostly in the occupied form, the uptake of apotransferrin being markedly smaller than metallotransferrin [19]. As demonstrated by Mladenovic, transferrin was needed for the appearance of aluminum toxicity on human erythroid [20]. Aluminum alone, even at very high levels, did not significantly affect erythroid colony growth. Moreover, inhibition

by aluminum was found to be directly related to the number of binding sites on transferrin, and was not observed in the presence of fully iron-saturated transferrin [20]. The scarce information available on the comparative uptake kinetics of iron-bound respect to aluminum-bound transferrin suggests that no significant differences in cellular uptake exist between both forms of metallotransferrin.

Cochran et al have found that human retyculocytes do not distinguish between aluminum-transferrin and iron-transferrin, and even that the aluminum transferrin metalloprotein complex is retained longer within these cells [21], thus generating a greater accumulation of aluminum transferrin than iron transferrin. Also, Romero et al postulated that the improvement of anemia observed with deferoxamine in dialysis patients with low aluminum levels may be due to the displacement of the aluminum molecules from transferrin and their replacement by iron [22]. Nowadays, with the use of rHuEPO, there is a greater consumption of the iron stores, the patients tend to decrease iron transferrin saturation, and therefore, they could offer a larger number of binding sites to form aluminum transferrin.

In the present set of patients, in the presence of aluminum overload more molecules of aluminum were probably offered by transferrin to the erythroid cells, reaching a proportion of approximately 1 to 2.4 aluminum to iron molecules. On the contrary, in baseline conditions, in the same patients the proportion of aluminum to iron on a molar basis was approximately 1 to 13.8. Moreover, the increased intraerythrocytic aluminum and the reduction in intraerythrocytic ferritin suggests the presence of a definite change in the uptake of these metallic elements by the erythroid cells. The decrease in the erythrocyte:plasma ferritin ratio in the patients with aluminum intoxication strongly suggests the existence of a depressed level of iron uptake, in spite of the normal/high size iron stores represented by the plasma ferritin. Even though the decrease in erythrocyte ferritin is not a measurement of the erythrocyte iron content, it should be accepted as representative of the amount of cell iron insofar as most of intracellular iron is complexed with ferritin.

The present data support previous experimental observations of decreased iron absorption and iron cellular uptake in aluminum overload, which raised the hypothesis that the microcytosis accompanying aluminum overload could be a significant form of iron deficiency, despite the existence of normal serum ferritin levels [23]. In agreement with the present results, the previous studies did not find differences in serum ferritin, but the degree of the anemia achieved was enough to stimulate the increase in serum transferrin [23]. By contrast, the present results did not favor the *in vitro* observation by Abreo, Glass and Sella of an increased iron uptake by Friend's erythroleukemia cells in the presence of aluminum [24], but they are in agreement with further data from the same authors, showing changes in iron compartmentalization and reduction in the cell content of ferritin [25].

As previously mentioned, aluminum produces some sort of interference in heme synthesis, which is mostly based in changes in the activity of enzymes involved in heme synthesis [7, 8, 11]. However, similar changes can also be found in classical iron deficiency [26]. Bia et al have postulated that the decreased red cell production and elevated red blood cell protoporphyrin in patients with modest degree of aluminum overload suggest that an effect in iron utilization is involved in aluminum-related anemia [11]. Nevertheless, these and other authors [7, 9, 11] hypothesized that this defect was not due to ferropenia, based in the finding of normal plasma ferritin and bone marrow staining for iron. As can be deduced from other studies [5, 6, 9] and from the present results, measurement of plasma ferritin alone may be insufficient to evaluate the real amount of iron which is being offered to the erythroid precursors. Therefore, though a direct toxic effect of aluminum on heme synthesis cannot be ruled out, our findings raise the possibility that aluminum-transferrin may convey the wrong metal to the erythrocyte precursors, providing an alternative pathogenetic explanation for the aluminum-related microcytosis.

Finally, another important message from our results is related to the epidemiological aspects of aluminum levels in dialysis fluids. We believe it is necessary to insist on the importance of very frequent, preferably monthly, controls of the quality of the water treatment in Dialysis Units aiming to achieve permanent levels of aluminum in the dialysis fluids lower than 5 μg/liter [27, 28]. This safety policy will decrease the always present possibility of aluminum intoxication. In this regard, moderate degrees of aluminum toxicity, as that described in the present study, can be easily and effectively prevented.

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Reprint requests to Dr. Jorge B. Cannata, Unidad de Investigación, Metabolismo Oseo y Mineral, Hospital Central de Asturias, Julián Clavería s/n, 33006 Oviedo, Spain.

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